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Phenylarsine Oxide Increases Intracellular Calcium Mobility and Inhibits Ca^{2+}-Dependent ATPase Activity in Thymocytes

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A rise in intracellular Ca^{2+} levels has been implicated as a regulatory signal for the initiation of lymphocyte proliferation. In the present study the mechanism underlying the elevation of $[Ca^{2+}]$ induced by phenylarsine oxide [PAO] was investigated in thymocytes. This agent inhibits HIV-1 replication and also NF-κB-mediated activation. It has been reported that the PAO-induced Ca^{2+} elevation results from an enhanced plasma membrane calcium permeability in T cells. Here, we present biochemical evidence that the PAO-induced Ca^{2+} increase was independent of external Ca^{2+}. Consistent with these facts, when $[Ca^{2+}]_i$ was depleted by prolonged incubation of the cells in Ca^{2+}-free medium, PAO addition did not lead to $[Ca^{2+}]_i$ increase. These data indicate the involvement of intracellular organelles of thymocytes as the source of Ca^{2+}. Moreover, evidence is presented that PAO inhibited Ca^{2+}-dependent ATPase activity from thymocytes and sarcoplasmic reticulum from skeletal muscle. This inhibition was dose-dependent, with a IC_{50} of about 30 μM for both preparations of enzyme. The ability of PAO to inhibit Ca^{2+}-dependent ATPase represents a novel mechanism of action for this drug. Present data suggest that the PAO-dependent $[Ca^{2+}]_i$ increase could be mainly the result of inhibition of Ca^{2+}-dependent ATPase. In addition, we describe also a Ca^{2+}-dependence for PAO effect on tyrosine phosphorylation. © 1999 Academic Press

Key Words: **phenylarsine oxide; calcium; thymocytes; Ca^{2+}-dependent ATPase.**

A variety of early biochemical events occurs upon antigen stimulation of T-cells (for review, see 1). The products of such processes comprise a wide array of molecules referred to as "second messengers." A rise of $[Ca^{2+}]_i$ has been implicated as a regulatory event during the initiation of lymphocyte proliferation (2). The exact role that Ca^{2+} might play in this cell activation process is unclear, but the possibility that Ca^{2+}/calmodulin could activate calcineurin or other Ca^{2+}-dependent proteins has been raised (3). In addition, clear evidence suggests that a network of tyrosine kinases and phosphatases also regulates early steps of T-cell activation (4). The requirement for phosphotyrosine phosphatase CD45 during the T-cell activation process agrees with a reciprocal interplay existing between intracellular kinases and phosphatases (5).

A group of chemical compounds (arsenoxides) acting on SH groups has been used as enzyme inhibitors. One of them, phenylarsine oxide (PAO), is a trivalent arsenical compound (6), which specifically inhibits tyrosine phosphatases in lymphocytes, while leaving $p56^{lck}$ and $p59^{fyn}$ protein tyrosine kinase activities intact (7). Also, PAO has been recently proposed to be a potential therapeutic drug, since it can inhibit HIV-1 replication in monocytic

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[2] Abbreviations used: PAO, phenylarsine oxide; NF-κB, nuclear factor κB; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; dbcAMP, dibutyryl 3′-5′-cyclic AMP; IP_3, inositol triphosphate.

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cell lines and also NF-κB-mediated activation (8). Evidence has been shown that PAO treatment of T lymphocytes (9) and Jurkat cells (10) inhibits the Ca^{2+} increase promoted by an antibody against the surface antigen CD3. However, the treatment of T cells with PAO alone has been observed to cause a dose-dependent increase in $[Ca^{2+}]_i$ that was not accompanied by a detectable increase in inositol 1,4,5-trisphosphate production (9,10). This dual effect does not seem related to its ability to inhibit CD45 phosphatase, since in CD45-mutant cells PAO also mobilizes Ca^{2+} (9). The present work was undertaken to analyze the intrinsic ability of PAO to increase $[Ca^{2+}]_i$ in lymphocytes. We present evidence that PAO exposure produced a rapid and sustained increase in $[Ca^{2+}]_i$ in rat thymocytes, which was independent of the presence of external Ca^{2+} in the medium. We also show that PAO inhibited Ca^{2+}-dependent ATPase activity from endoplasmic reticulum of thymocytes and muscle in a dose-dependent manner. These results suggest that PAO increases $[Ca^{2+}]_i$ in lymphocytes through an acute arrest of the endoplasmic reticulum Ca^{2+} pump activity, similar to the effect previously described for thapsigargin (11,12). We thus provide the first evidence that PAO-induced Ca^{2+} mobilization may be caused by the inhibition of microsomal Ca^{2+}-dependent ATPase.

MATERIALS AND METHODS

Materials

PAO and Fura-2 acetoxymethyl ester (Fura-2 AM) were obtained from Sigma. PAO was dissolved in DMSO, and the concentration of the solvent in the cell culture never exceeded 0.05% (v/v).

Preparation of Thymocytes and Subcellular Organelles

Male mice weighing 25–35 g were used. Thymocytes were isolated as described earlier (13). In some experiments, in order to deplete intracellular Ca^{2+} stores, a fraction of thymocytes was treated with 5 mM EGTA in Krebs Ringer bicarbonate (KRB) buffer at 37°C for 30 min. After being washed, untreated or treated cells were incubated (10×10^6 cells/ml) in KRB with the additions indicated in the figure legends. The cell viability was assessed by determining the percentage of cells that excluded Trypan blue. Microsomes from thymus and sarcoplasmic reticulum from skeletal muscle were prepared basically as described (14,15). Briefly, thymuses and skeletal muscles from rat were dissected, washed in 0.9% (w/v) NaCl, trimmed, and homogenized (1:5, w/v) in a cold buffer containing 20 mM Tris–HCl, 1 mM EDTA, and 250 mM saccharose, pH 7.4. The homogenate was centrifuged at 1600*g* for 10 min, and the supernatant obtained was centrifuged again at 12,000*g* for 30 min. The pellet was discard and the supernatant was centrifuged again at 100,000*g* for 1 h and the resulting pellet (microsomes/sarcoplasmic reticulum) was resuspended in the stock buffer using a Teflon–glass homogenizer. The purity of microsomes/sarcoplasmic reticulum preparations was analyzed by specific enzymatic markers, as indicated previously (14,15). Protein concentration was estimated by a modified Lowry method (16).

Measurement of $[Ca^{2+}]_i$

Cell loading with Fura-2 AM and measurement of $[Ca^{2+}]_i$ were performed basically as in (17), with the modifications previously described (14). Briefly, thymocytes (6×10^7/ml) were incubated with 2 μM Fura-2 AM in a medium composed of 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 2.5 mM probenecid, 10 mM glucose, 1 mg/ml bovine serum albumin (BSA), and 25 mM Hepes, pH 7.4. After 30–45 min at 37°C, the cells were washed and resuspended in the same medium but lacking Fura-2 AM. Calibration was made by lysing the cells with 50 μM digitonin followed by quenching the fluorescence with 3 mM Mn^{2+}, and Fura-2 leakage was determined by the decrease in fluorescence taking place after the addition of 100 μM Mn^{2+}. Experiments were performed using 0.6 ml of the cell suspension, placed in a thermostatically controlled holder, and fluorescence was measured using a Perkin–Elmer spectrofluorimeter (LS-5). Fura-2 fluorescence (λ_{exc} at 340 nm and λ_{emi} at 500 nM) was continously recorded.

Ca^{2+}-Dependent ATPase Activity

ATPase activity from microsomes/sarcoplasmic reticulum was determined by the measurement of release of P_i (18). The reaction mixture contained 50 mM KCl, 20 mM Mops (pH 6.8), 10 mM $MgCl_2$, 100 μM $CaCl_2$, 20 μM ionomicin, and 1–1.5 mg/ml of protein. The mixture was preincubated for 5 min at 37°C with different concentrations of PAO ranging from 5 to 100 μM. The reaction was initiated by the addition of 0.5 mM ATP, incubated for 3 min, and

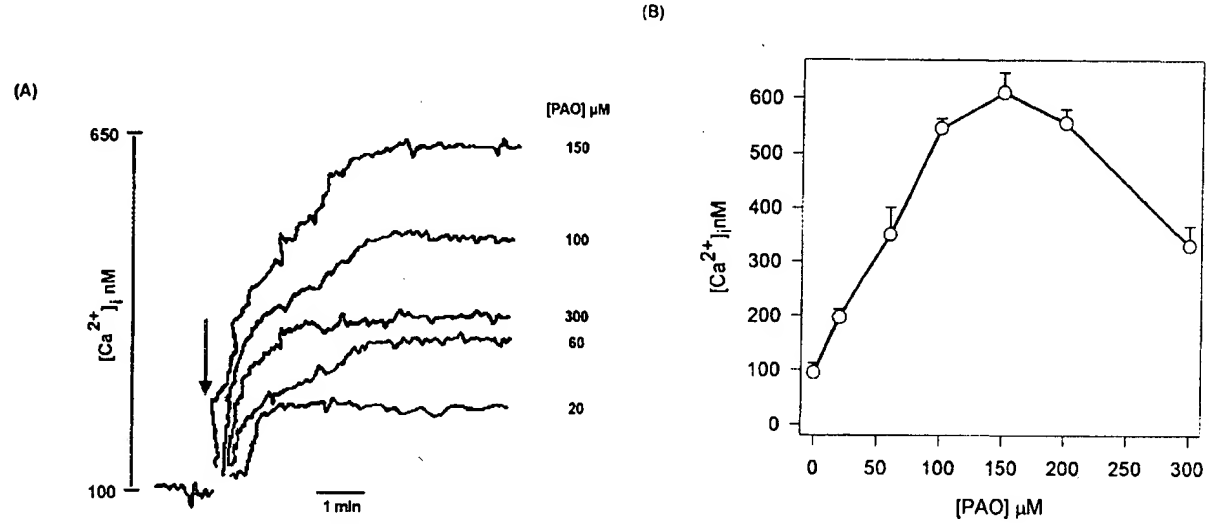

FIG. 1. Time-course (A) and dose-effect (B) of PAO on intracellular Ca^{2+} levels. (A) After loading with Fura-2, thymocytes were suspended in medium containing 1 mM Ca^{2+} and the change in fluorescence was recorded during 10 min. PAO was added at the indicated time (arrow) at the concentration shown at the right of each trace. A representative experiment from three independent measurements is shown. The range of variation between groups of experiments was less than 10%. (B) The values of $[Ca^{2+}]_i$ measured 6 min after PAO addition are plotted as a function of the PAO concentration. Values are means ± SE of three independent experiments.

stopped with molybdate/ascorbic acid reactive solution. The absorbance at 630 nM was read after 30 min. Basal, Ca^{2+}-independent ATPase activity was similarly measured, but in the presence of 0.5 mM EGTA and without added calcium. The subtraction of this last value from that obtained in the presence of calcium corresponds to the Ca^{2+}-dependent ATPase activity.

Electrophoresis, Immunoblotting, and Detection of Protein Tyrosine Phosphorylation

In the experiment described in Fig. 5, the reaction was terminated after 15 min by centrifugation and aspiration of the reaction medium. A volume (90 μl) of lysis buffer containing 50 mM HCl/Tris, pH 7.6, 1% Triton X-100, 300 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml of aprotinin and leupeptin, 50 μM PAO, and 20 mM NaF was added to the pellet. The cell lysates were resolved by SDS/10%–PAGE by standard procedure as detailed recently (19). Membrane was immunoblotted with anti-phosphotyrosine monoclonal antibody (4G10) (1:2000 final dilution). Bound antibodies were detected by luminol enhanced chemiluminiscence, using 4-iodophenol as the enhancer of the chemiluminesce reaction as described by us (19).

RESULTS

Levels of Intracellular Calcium

Treatment of thymocytes with different doses of PAO, in an external medium containing 1 mM Ca^{2+}, produced an elevation of $[Ca^{2+}]_i$ from basal values (about 90–110 nM) to 660 nM. This increase needed 2–4 min to reach a plateau and remained stable for at least 8 min (Fig. 1A). The effect of PAO on $[Ca^{2+}]_i$ was dose-dependent and reached its maximum level at 150 μM. Higher concentrations of PAO did not produce a further increase of $[Ca^{2+}]_i$ or even decreased it. The half-maximally effective dose for PAO was about 50 μM (Fig. 1B). This pattern, and also the required PAO concentrations, was similar to those previously described for lymphocytes and Jurkat cells (9,10). Similar conditions of incubation showed that PAO at 100 μM had no effects on thymocyte viability (measured on the basis of lactic dehydrogenase release) after 1 h of treatment (data not shown). When PAO was added to the cells in nominally Ca^{2+}-free incubation medium (i.e., without added Ca^{2+} and with 5 mM EGTA], a rapid transient increase in $[Ca^{2+}]_i$ was observed (Fig. 2A). When the preincubation of thymocytes in Ca^{2+}-free medium was continued for 5, 10, or 15 min (before PAO addition), a progressive reduction in the PAO-dependent $[Ca^{2+}]_i$ increase was recorded (Figs. 2B–

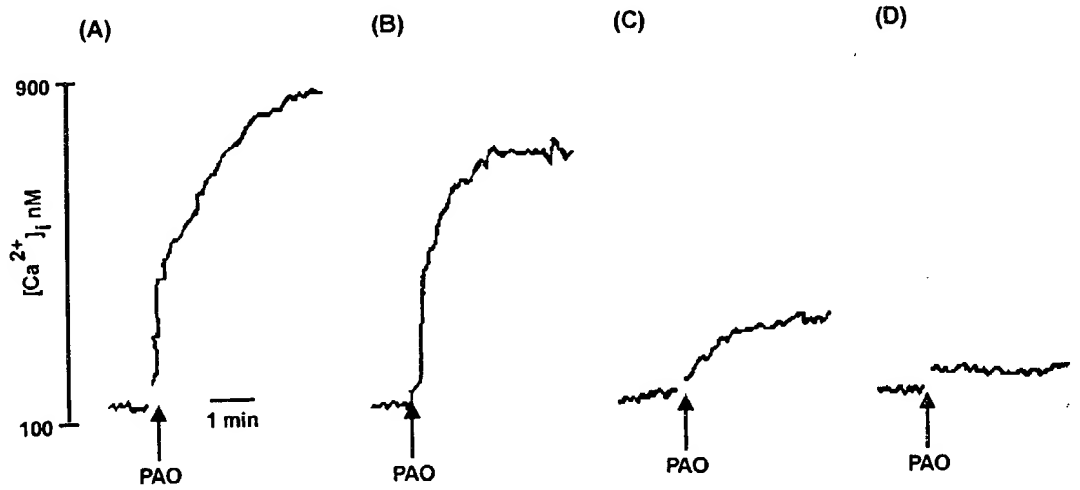

FIG. 2. Effect of preincubation with EGTA on PAO-stimulated $[Ca^{2+}]_i$ levels. Thymocytes were loaded with Fura-2. Before PAO addition, the cells were pretreated in a medium where the extracellular Ca^{2+} was chelated by the addition of 5 mM EGTA for 0 min (A), 5 min (B), 10 min (C), or 15 min (D). At these times, 150 μM PAO was added to the cell suspension, and changes in fluorescence were recorded. Similar traces were observed in three separated experiments.

2D). These data indirectly suggest that the PAO-induced $[Ca^{2+}]_i$ increase was due to the release of Ca^{2+} from intracellular stores, since when the cells were depleted of Ca^{2+} (i.e., after 15 min incubation with 5 mM-EGTA), PAO was unable to mobilize this cation. The further addition of external Ca^{2+} to these EGTA-treated cells rapidly produced an influx of extracellular Ca^{2+} inside the cells (data not shown). In order to further analyze the source of the PAO-induced $[Ca^{2+}]_i$ increase, experiments using $NiCl_2$, a well-known blocker of Ca^{2+} transport through the plasma membrane (20), were performed. The presence of 400 μM Ni^{+2} in the incubation medium did not produce any detectable effect on the PAO-induced $[Ca^{2+}]_i$ increase (Fig. 3A). This result further confirmed the previous assumption that observable PAO-induced $[Ca^{2+}]_i$ increase arose from intracellular stores. In a search for other inhibitors of tyrosine phosphatases which could produce a similar action on $[Ca^{2+}]_i$ levels, we observed that vanadate was without effect (data not shown). It has been previously described that dibutyryl-cyclic AMP (dbcAMP), as well as agents which increase intracellular cAMP (e.g., forskolin and prostaglandin E_2), suppresses in a dose-dependent fashion the anti-CD3-induced Ca^{2+} elevation in T lymphocytes (21). These data prompted us to study whether dbcAMP was able to inhibit the PAO-induced $[Ca^{2+}]_i$ increase in thymus cells. Figure 3B illustrates that the presence of 1.5 mM dbcAMP abolished almost completely (80–90% inhibition) the $[Ca^{2+}]_i$ increase stimulated by different doses of PAO. It is noteworthy that at the analyzed dose of PAO a small fraction of PAO-induced $[Ca^{2+}]_i$ increase remained not inhibited by dbcAMP. The inhibitory effect of dbcAMP was not canceled by PAO concentrations higher than 150 μM and remained constant during the time measurement (8–10 min). A similar inhibitory effect was observed when the cells were previously preincubated for 1–3 h with dbcAMP (data not shown). The mechanism by which cyclic AMP inhibits the agonist-dependent Ca^{2+} mobilization remains to be clarified, and it could be related to a yet undetermined protein kinase.

Inhibitory Effect of PAO on Ca^{2+}-Dependent-ATPase Activity

In order to establish the mechanism underlying the PAO-induced $[Ca^{2+}]_i$ increase, and since the pattern of this phenomenon was found to be very comparable to that described for thapsigargin, we tested the possibility that the $[Ca^{2+}]_i$ increase could be due to some alteration of Ca^{2+}-dependent ATPase activity. Previously it had been demonstrated that thapsigargin pretreatment of rat liver microsomes blocked subsequent loading with $^{45}Ca^{2+}$ and also produced a rapid inhibition of Ca^{2+}-dependent ATPase activity (11). Also thapsigargin elicited a rapid and dose-dependent increase in $[Ca^{2+}]_i$ (12). Figure 4 illustrates the effect of PAO on different preparations of Ca^{2+}-dependent ATPases. The direct measurement of enzymatic ATP hydrolysis revealed that

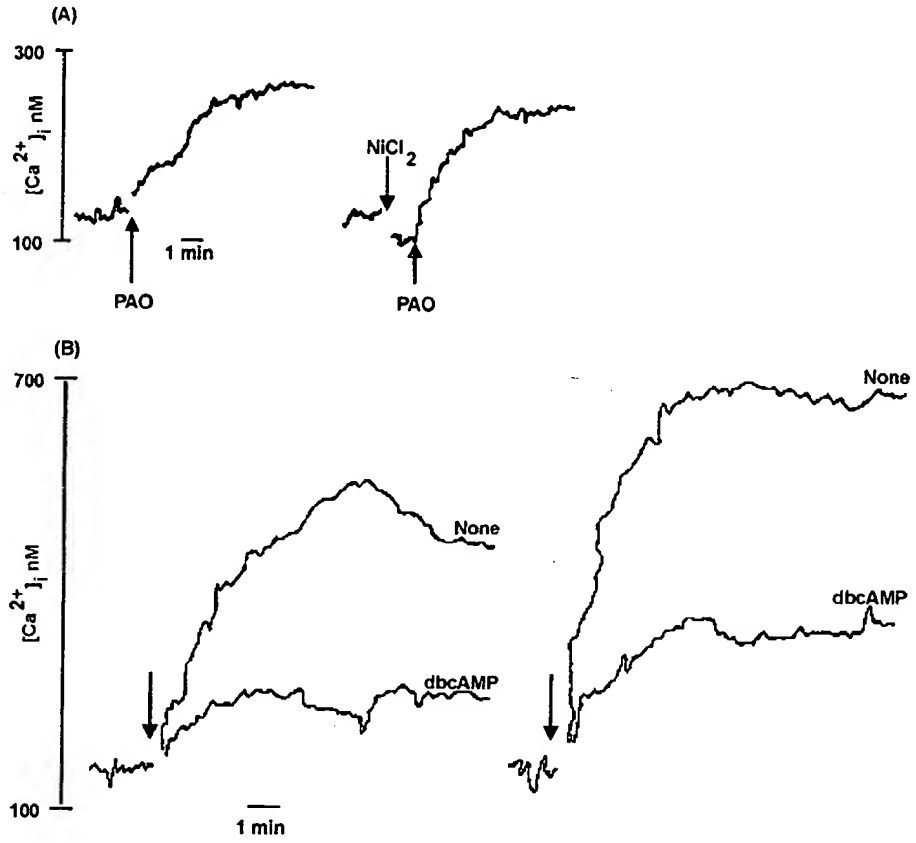

FIG. 3. Effects of $NiCl_2$ (A) and dibutyryl-cyclic AMP (B) on PAO-stimulated $[Ca^{2+}]_i$ levels. (A) Fura-2-loaded thymocytes were pretreated for 1 min either in the absence (left) or in the presence (right) of 400 μM Ni^{+2}, an inhibitor of calcium transport through the plasma membrane. Thereafter, lymphocytes were treated with 60 μM PAO (arrow), and the change of fluorescence was recorded. (B) Lymphocytes were preincubated in the absence (none) or in the presence (dbAMP) of 2 mM dibutyryl-cyclic AMP for 10 min. Thereafter, PAO (arrow) at 100 mM (left) or at 150 μM (right) was added to cell suspension and the fluorescence was recorded.

PAO inhibited both microsomes/sarcoplasmic reticulum Ca^{2+}-dependent ATPase with an EC_{50} value of about 30 μM (Fig. 4). Nevertheless, basal Ca^{+2}-independent ATPase was unaffected after PAO treatment (data not shown).

Intracellular Ca^{2+} and PAO-Dependent Tyrosine Phosphorylation

Since PAO increases both intracellular Ca^{2+} levels and tyrosine phosphorylation, acting as a potent inhibitor of tyrosine phosphatases (7,9,10), we have analyzed the potential relationship between Ca^{2+} levels and PAO-dependent tyrosine phosphorylation. For this purpose, we have depleted Ca^{2+} from the internal stores by preincubating the cells with EGTA, and after being washed, PAO was added to the cells at the indicated dose for 15 min (Fig. 5). It was observed that Ca^{2+}-depleted cells present a lower level of tyrosine phosphorylation in most proteins than untreated cells. These results suggest that intracellular Ca^{2+} may be required either for the PAO-dependent inhibition of tyrosine phosphatase or for the activation of the corresponding tyrosine kinase. The absence of Ca^{2+} seems especially relevant for tyrosine phosphorylation of proteins with apparent molecular masses of 129, 112, and 56 KDa (Fig. 5). In fact, although the addition of PAO allows the tyrosine phosphorylation of proteins, it is noteworthy that, at the same PAO concentration used, the presence of Ca^{2+} induced the tyrosine phosphorylation of specific proteins in a greater extent than its absence. Although the increase of PAO-dependent tyrosine phosphorylation is well known for other cellular systems (7,9,10), the Ca^{2+} dependence for PAO effect has not been previously reported.

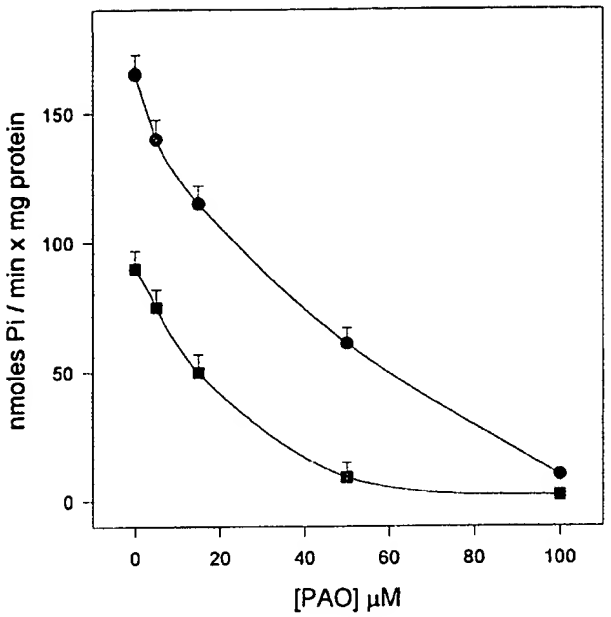

FIG. 4. Ca^{2+}-dependent ATPase(s) inhibition by PAO. Microsomes for thymus (■) and muscle sarcoplasmic reticulum (●) were prepared from mice as indicated under Materials and Methods. These subcellular preparations were incubated at 37°C with the indicated concentrations of PAO for 5 min. The reaction was initiated by the addition of 0.5 mM ATP. After 3 min of incubation, phosphate from the medium was analyzed. Data are the means of three separate experiments performed in triplicate.

DISCUSSION

The identification of PAO as a specific inhibitor of tyrosine phosphatase makes this compound an important tool for analyzing the intracellular signal transduction process. The wide variety of PAO effects on biological systems includes the inhibition of NADPH-oxidase from macrophages and neutrophils (22,23), tyrosine phosphorylation of phospholipase Cγ-2 (24), TNF-dependent activation of NF-κB (25), and the HIV-1 replication (8).

Present data confirm the previously described PAO-induced $[Ca^{2+}]_i$ increase effect in lymphocyte cell lines (10,11,26), extend these observations to a primary cell preparation (such as thymocytes), and offer a mechanism for its interpretation. Mobilization of Ca^{2+} frequently exhibits two phases, an initial peak due to its release from intracellular stores and a second phase attributable to an influx of Ca^{2+} through the plasma membrane, termed capacitative entry (27). But, at a difference with previous data (10,11,26), our results implicate Ca^{2+} released from internal stores as the main component of the observed cytosolic Ca^{2+} elevation in thymocytes. This assumption is based on the following observations: first, PAO raised $[Ca^{2+}]_i$ in the absence of extracellular calcium (Fig. 2A). Furthemore, Ni^{2+}, a blocker of Ca^{2+} influx through the plasma membrane, did not alter the PAO-effect on $[Ca^{2+}]_i$ (Fig. 3A), discarding the idea that an effect of membrane permeability to this cation could play some role in the PAO-induced $[Ca^{2+}]_i$ increase. And third, when stringent experimental conditions (15 min of treatment with 5 mM EGTA) were introduced to deplete intracellular Ca^{2+} stores, PAO was unable to elicit any $[Ca^{2+}]_i$ increase (Fig. 2D). Previous experiments on lymphocytes (9,10) and on endothelial cells (26), using mild Ca^{2+} depletion conditions (i.e., 1.5 mM EGTA treatment during 2 min), showed a partial inhibition of the PAO-dependent Ca^{2+} increase, and the authors concluded that PAO also stimulates Ca^{2+} influx from the extracellular milieu. However, it may be argued that this effect could be the result of a decreased Ca^{2+} reserve in the internal stores, without the participation of Ca^{2+} influx through the plasma membrane. In fact, we have found (Figs. 2A–2D) a gradual decrease on $[Ca^{2+}]_i$ to occur depending on the period of EGTA treatment, similar to their finding using mild conditions. The potential stimulation by PAO of Ca^{2+} influx through the plasma membrane is difficult to prove using external EGTA, since in such nominally Ca^{2+}-free medium the depletion of inter-

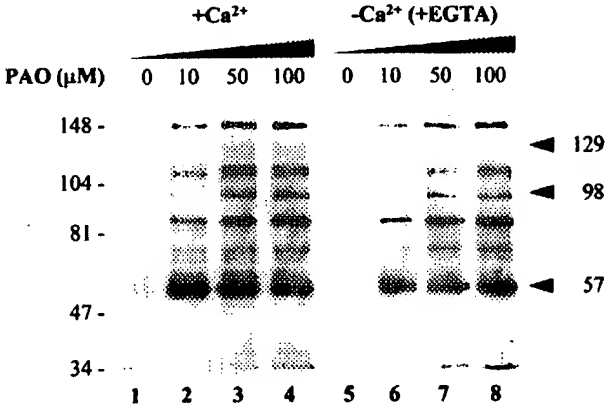

FIG. 5. Effect of PAO on tyrosine phosphorylation by calcium-depleted cells. Control, untreated thymocytes (lanes 1–4), and thymocytes exposed to 5 mM EGTA for 30 min (lanes 5–8) were washed and incubated either without additions (lanes 1 and 5) or with 10 μM (lanes 2 and 6), 50 μM (lanes 3 and 7), and 100 μM (lanes 7 and 8) of PAO for 15 min. After lysis, proteins were separated by SDS–PAGE and phosphorylation on tyrosine was analyzed by immunoblotting with anti-phosphotyrosine mAb (4G10). Two experiments were performed with similar results.

nal Ca^{2+} stores provokes a sustained and agonist-independent Ca^{2+} influx when external Ca^{2+} is added (capacitative entry) (28, and data not shown).

Ca^{2+} pumps are members of a large superfamily of P-type ATPases and have been classified as endoplasmic reticulum or plasma membrane type Ca^{2+}-ATPase (types IIA and IIB, respectively), based on enzymes first identified in animal systems (29,30). We show evidence for the first time that PAO inhibited the Ca^{2+}-dependent ATPase from thymus microsomes and sarcoplasmic reticulum (Fig. 4), suggesting that the PAO-induced $[Ca^{2+}]_i$ elevation could be the result of a predominant balance toward its extrusion from Ca^{2+} stores. This effect is attributable to the inhibition of cytosolic Ca^{2+} uptake, which is dependent on Ca^{2+}-ATPase from endoplasmic reticulum. In agreement with this possibility, evidence has been presented that PAO does not induce phosphoinositide hydrolysis with the concomitant production of $InsP_3$ (9,26,31). Although $InsP_3$ is clearly a major regulator of intracellular Ca^{2+} handling (32), a variety of experimental evidences suggest that $InsP_3$-independent Ca^{2+} regulatory pathways exist as well. For example, resting B-cells stimulated with concanavalin A display a large increase in intracellular Ca^{2+} levels, but only a very small release of $InsP_3$ (33). Likewise, a dichotomy between Ca^{2+} and phosphoinositide hydrolysis has been noted in T-cells (31). The effects described here for PAO are similar to those previously reported for thapsigargin regarding promotion of an IP_3-independent Ca^{2+} increase (34) and inhibition of Ca^{2+}-ATPase (11,12), although the effect elicited by PAO seems less potent and less specific for microsomes than that of thapsigargin. The activity of PAO as an inhibitor of tyrosine phosphatases (7) could be causally related to its ability to inhibit Ca^{2+}-dependent ATPase activity reported here. In this context, the plasma membrane Ca^{2+} pump is sensitive to the cyclic GMP-dependent protein kinase (35), and the sarcoplasmic reticulum Ca^{2+}-ATPase activity is also regulated by a kinase system (36). Interestingly, Ca^{2+} levels may be directly regulated by protein tyrosine kinases in T-lymphocytes (31) or endothelial cells (26), and it has also been described that phosphorylation down-regulates the store-operated Ca^{2+} entry pathway (37).

Therefore, given the potential ability of PAO to interact with the Ca^{2+} pump, this compound may constitute a suitable molecular tool for analyzing Ca^{2+} homeostasis and offer new insight into the Ca^{2+}-induced activation of transcription factors, such as NF-κB and NF-AT (38).

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